

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
9 August 2001 (09.08.2001)

PCT

(10) International Publication Number  
**WO 01/56628 A1**

(51) International Patent Classification<sup>7</sup>: **A61L 27/34**,  
31/10, 27/32, 31/08

(21) International Application Number: PCT/NL01/00071

(22) International Filing Date: 1 February 2001 (01.02.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
00200393.7 4 February 2000 (04.02.2000) EP

(71) Applicant (for all designated States except US): ISOTIS  
N.V. [NL/NL]; Prof. Bronkhorstlaan 10, NL-3723 MB  
Bilthoven (NL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **LAYROLLE,**  
**Pierre, Jean, François** [FR/NL]; Kruisweg 20 bis,  
NL-3513 CT Utrecht (NL). **LIU, Yuelian** [CN/NL];  
Eligenstraat 39, NL-3512 PT Utrecht (NL).

(74) Agent: **PRINS, A., W.**; Vereenigde, Nieuwe Parklaan 97,  
NL-2587 BN The Hague (NL).

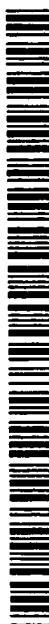
(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,  
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,  
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.



**WO 01/56628 A1**

(54) Title: PROTEINACEOUS COATING

(57) Abstract: The invention relates to a method for providing a proteinaceous coating on a medical implant, comprising the steps of: submersing the implant in a first aqueous solution comprising a protein and magnesium, calcium and phosphate ions through which a gaseous weak acid is passed; degassing the solution; allowing a coating to precipitate onto the implant; submersing the coated implant in a second solution to redissolve the magnesium, calcium and phosphate ions and to obtain the proteinaceous coating.

Title: Proteinaceous coating

The invention relates to the field of medical implants. More in particular, the invention relates to a coating which improved the biocompatibility and bone-bonding properties of medical implants, such as orthopedic and dental  
5 prostheses.

Recently, a biomimetic coating has been developed for coating medical implants with ceramic materials, such as bone-like hydroxyapatite. This technology has been disclosed in European patent application 98203085.0 and comprises  
10 soaking an implant material, e.g. a scaffold for tissue engineering bone, into a super saturated calcium phosphate solution resembling a physiological fluid. A calcium phosphate layer uniformly precipitates on the implant surface under modulated nucleation and crystal growth conditions.  
15 This method mimics the way hydroxyapatite bone crystals are formed in the body. Considering the physiological conditions under which the biomimetic coating is grown from a fluid at body temperature, biologically active agents, such as antibiotics, can be coprecipitated.

20 In EP 0 806 212, an implantable coated device is disclosed that comprises a calcium phosphate layer, in which a biologically active substance may be incorporated. The coating comprising calcium phosphate and a growth factor can give rise to an enhancement of bone formation. For the  
25 preparation of the coating the surface of the device needs to have a roughness(Ra value) 10-1,000 nm.

WO 97/41273 describes a process for coating a metallic or ceramic substrate by heating a certain mineral solution, in which the substrate is immersed, to a high  
30 temperature until the pH is at least 8, after which deposition of crystalline carbonated hydroxyappetite is on the substrate is induced. Such a coating is reported to be osteoinductive, but the incorporation of bioactive agents is not disclosed. It is unlikely that the process would be  
35 suitable for such purpose, since the combination of a high

temperature and a high pH, would have a detrimental effect on the thermosensitive bioactive agents.

Many mineralized tissues in living organisms are composed of crystals formed under well-controlled conditions. Proteins are key participants in the control process. Some proteins envelop the individual crystals, whereas others are occluded inside the crystals. How these proteins become occluded inside a crystal and what their role is in the crystallization process and in the determination of the properties of the crystal still remains unclear.

The present invention seeks to find a way of using the expected controlling function of proteins in mineralization processes. In particular, it is an object of the invention to find a way of using a protein to induce mineralization, calcification and/or the formation of bone tissue on a medical implant.

These objects, as well as other objects of the invention that will become clear from the present description, have been achieved by virtue of a proteinaceous coating to the implant which is applied thereto in a specific manner. Accordingly, the invention relates specifically to a method for providing a proteinaceous coating on a medical implant, comprising the steps of:

- submersing the implant in a first aqueous solution comprising a protein and magnesium, calcium and phosphate ions through which a gaseous weak acid is passed;
- degassing the solution;
- allowing a coating to precipitate onto the implant;
- submersing the coated implant in a second solution to redissolve the magnesium, calcium and phosphate ions and to obtain the proteinaceous coating.

Surprisingly, it has been found that a proteinaceous coating may be provided on a medical implant, which coating induces nucleation and growth of calcium phosphate crystals, both *in vitro* and *in vivo*. Although the coating itself will typically not contain any calcium phosphate material, it has

been found to act as a type of template or matrix for mineralization. This advantageous property allows for the application of the medical implant to serve as a scaffold for tissue engineering bone tissue.

5           On the other hand, said property of course also increases the suitability of the implant for the purpose it originally had, i.e. being implanted in a patient in need of a bone substitute. The proteinaceous coating described herein can induce deposition of a variety of calcium phosphate  
10 compounds containing carbonate and others ions on the surface of an implantable device. The layers will be similar in composition and crystallinity with bone and teeth minerals and have desired bioresorbability, bone-bonding properties to improve the biological fixation of medical devices to living  
15 calcified tissue.

          The proteinaceous coating may further form a composite with calcium phosphate crystals, for instance in vivo, leading to a biomimetic coating with mechanical properties superior to those of conventional ceramic  
20 coatings. It is believed that the protein may function as a reinforcement of a biomimetic coating by bonding calcium phosphate crystals together.

          Furthermore, the proteinaceous coating enhances attachment of cells and improves the biocompatibility and  
25 bone-bonding properties of medical implants.

          The medical implant on which a coating is applied in accordance with the invention may be of any inorganic, metallic, polymeric or organic material. The implant may be flat, dense or of a complex shape. It may have a porous,  
30 beaded or meshed ingrowth surface.

          Metals, such as stainless steel, titanium, nickel, cobalt, chrome, niobium, molybdenum, zirconium, tantalum, and combinations thereof, can be coated for orthopaedic and dental applications. For example, devices used in total hip  
35 arthroplasty such as porous or non-porous acetabular cups and the proximal region of hip stems may be coated.

Ceramic materials, such as alumina and zirconia, glasses such as bioactive glasses made of  $\text{CaO-SiO}_2\text{-P}_2\text{O}_5$ , and calcium phosphates, such as hydroxyapatite and tricalcium phosphate, may be coated.

5           The subject coatings can be applied to various polymers and plastics, more preferably biocompatible or bioresorbable ones like polyactive™, a copolymer of polyethylene glycol and polybutylene terephthalate.

          Before applying the coating, the substrates are  
10   preferably cleaned or treated to remove any surface contaminants and to promote good adhesion of the coating. Various methods for cleaning may be employed. The metallic implants may be rinsed with a degreaser, i.e. acetone, alkyl alcohols, etc. and then thoroughly rinsed with pure water.

15           In order to improve coating adhesion, various surface treatments may be applied to metal implants. Mechanical surface treatments, such as sand-blasting, scoring, polishing and grinding can increase surface roughness of the implants and improve the bonding strength between the coatings and  
20   substrate. For similar purposes, chemical surface treatments may be also applied to metal substrates prior to coating. Among others chemical treatments available for metals, acid etchings will be preferred by treating implantable devices with strong mineral acids, such as hydrofluoric,  
25   hydrochloric, sulfuric, nitric and perchloric acids. It may also be useful to treat the metal devices with oxidizing agents such as nitric acid, peroxyhalogen acids, hydroxyperoxides, or hydrogen peroxide to form a fresh metal oxide layer. After the mechanical or chemical treatment, it is necessary  
30   to rinse the implants with pure water under ultrasounds for removal of surface contaminants.

          The method for coating medical implants consists of soaking medical implants into a calcifying solution comprising a protein at low temperature. This simple method  
35   is based on the finding that calcium phosphates are more soluble in mildly acidic medium than at neutral and even

basic pH. This also applies at conditions which essentially do not affect the stability and activity of the protein in a harmful way. Thus, aqueous solutions of calcium and phosphate ions and a protein can be more concentrated at mildly acid  
5 than at neutral pH. In other words, calcium phosphates precipitate at neutral or basic pH while they remain soluble at mildly acidic pH from a solution having the same concentrations of salts.

An increase of pH in the solution can induce the  
10 following stages: under-saturation, super-saturation or the formation of a meta-stable state, nucleation and crystal growth. Calcium phosphate nuclei can form onto a substrate - heterogeneous nucleation- when a solution has reached the super-saturation limit or the meta-stable state. At the  
15 super-saturation state, crystals can subsequently grow from metastable fluids. At higher saturation, homogeneous nucleation or precipitation in the solution is the predominant process. This invention makes use of pH changes to control the above stages and to induce the deposition of  
20 carbonated calcium phosphate layers on the surface of medical implants.

The above object can be achieved by bubbling a gaseous weak acid, preferably carbon dioxide gas, into a calcifying solution in order to decrease pH and thereby to  
25 increase the solubility of calcium phosphate salts. It is well known that natural sparkling water has a mildly acidic pH resulting from dissolved carbon dioxide gas. It is also an important feature that the pH of mineral water slowly increases to neutral or slightly basic pH during the natural  
30 release or exchange of dissolved carbon dioxide gas with air.

In a number of preferred embodiments, the bubbling of carbon dioxide gas into the calcifying solution is required. Carbon dioxide gas will dissolve in the calcifying solution and form hydrogen carbonate ions in water. The said  
35 medical implants are placed into an aqueous calcifying solution in which a gaseous weak acid, such as carbon dioxide

gas, is passed through to produce a weakly acidic media. The initial pH of said calcifying solution is maintained in the range 3-7, preferably about 5.5 to 6.5 by bubbling CO<sub>2</sub> gas. The carbon dioxide gas is introduced into the solution at a  
5 sufficient pressure to continuously generate bubbles. The pressure of CO<sub>2</sub> gas will be in the range 0.1-10 bars, preferably within 0.5 to 1.5 bars, more preferably about 1 bar.

In a method according to the invention, the presence  
10 of magnesium, calcium and phosphate ions in the calcifying solution is essential. Particularly, the presence of magnesium has been found to be important for controlling the crystal growth of the coating during deposition from the calcifying solution. An optimum control of crystal growth  
15 leads to a uniform, strong and wear resistant coating. Particularly, the attachment of the coating to the substrate is beneficially effected by the presence of magnesium ions in the calcifying solution. A coating prepared according to the invention, preferably has crystals having a size in the  
20 submicrometer range. In a preferred embodiment, additional inhibitors of crystal growth, such as carbonate ions, may be incorporated in the calcifying solutions. If required, counter ions, like sodium and chloride might also be present to provide a constant ionic strength.

25 Preferably, the calcifying solution is prepared while the gaseous weak acid is bubbled through, in order to avoid precipitation. The introduction of the gas decreases the pH of the solution and allows the complete dissolution of the magnesium, calcium and phosphate, and possible other salts.  
30 Preferably, the bubbling is started at least 5 minutes before, and during, the addition of the salts. Thus, the pH is lowered to approximately 3-8, more preferably to 5.5-6.

Of course it is also possible to start the bubbling with the gaseous weak acid after the addition of the desired  
35 amounts of the salts to the solution. Once the bubbling is

started, in accordance with this embodiment, it is important to ensure that the salts dissolve completely.

The calcifying solution is preferably prepared with ultra pure water and pure grade chemicals. The calcifying solution is preferably filter sterilized through a 0.2 microns filter membrane prior to use. The molar calcium to phosphorus ratio in the calcifying solution is generally within the range 1-3, more preferably between 1.5 to 2.5. The concentrations of the ions in the calcifying solution are chosen such, that in the absence of the gaseous weak acid, the solution is super-saturated or oversaturated. The molarity of the calcium source will generally be in the range 0.5-50 mM, preferably about 2.5 to 25 mM. The phosphate source will generally be from about 0.5 to 20 mM, more preferably about 1 to 10 mM. The concentration of magnesium in the calcifying solutions will usually be within the range 0.1-20 mM, more preferably about 1.5 to 10 mM. The carbonate concentration will range from 0 to 50 mM, more preferably 0 to 42 mM. The ionic strength will be within the range 0.10-2 M, more preferably in between 0.15 to 1.5 M. The calcifying solution is preferably stirred to approximately 10-1000 rpm, more usually 50 to 200 rpm. The temperature is maintained at about 5-80°C, preferably in the range of about 5-50°C.

The protein is preferably present in the above described calcifying solution. It may be added before, during or after the dissolution of the various ions that are desired in the coating. The concentration in which the protein is preferably present in the calcifying solution preferably lies between 0.001 and 10 g/l, more preferably between 0.01 and 1 g/l.

In principle, the present method may be carried out using any type of protein. Preferred, highly suitable proteins are electronegatively charged at the pH at which precipitation occurs. It is further preferred that the solubility of the protein is at least 1 gram per liter water at neutral pH. In addition, it is considered advantageous if



the protein possesses disulfide bridges in its structure. Examples of highly suitable proteins include albumin, casein, gelatin, lysozyme, fibronectin, fibrin and chitosan. In principle, any protein based on amino acids which have an isoelectric point below 7 and are negatively charged may be used. Examples of such amino acids include alanine, aspartic acid, cysteine, glutamine, glycine, isoleucine, leucine, methionine, proline, phosphoserine, serine and valine. Particularly preferred proteins are synthetic proteins, such as polylysine, polyalanine, and polycysteine. Biologically active proteins, such as growth factors (e.g. BMP, FGF, TGF), may also be advantageously used.

Optionally, the proteinaceous coating may be bound to the implant covalently by making use of a coupling or linking agent. This agent should be capable of reacting with OH groups on the implant surface and with amino groups of the protein. The covalent bond will ensure a good mechanical attachment of the proteinaceous coating to the implant. The coupling agent is preferably chosen from the group of isocyanates, cyanuric acid, titanium alkoxides ( $\text{Ti}(\text{OR})_4$ ), and silicon esters, such as  $\text{Si}(\text{OR})_2\text{Cl}_2$ , wherein R represents an alkyl group of 1-4 carbon atoms. In order to obtain the covalent linkage, the coupling agent is simply added to the calcifying solution in an appropriate amount which will typically lie between 1 and 20 wt.%, based on the weight of the solution.

The carbon dioxide has a limited solubility in aqueous solutions. In contact with air, a carbonated aqueous solution is free of  $\text{CO}_2$  or completely degassed within few hours depending on the surface of solution in contact with air. The complete exchange of dissolved  $\text{CO}_2$  gas with atmosphere may be performed in approximately 8 to 48 hours, more preferably between 12 to 24 hours. The natural release of  $\text{CO}_2$  gas causes the pH of the remaining solution to increase. In other words, saturation in the calcifying solution can increase until the precipitation of the

bioactive layers, including calcium phosphate and protein, on the surface of implantable materials occurs. Optionally, air can be bubbled through the solution to degas or aerate the solution and accelerate the escape, release or exchange of the gaseous weak acid. The initial and final pH values as well as pH changes with time depend on the amount of carbonate and phosphate salts added to the calcifying solution. The buffering capability can be adjusted to a desired pH value by adding more or less of phosphate and carbonate salts. The pH can be maintained within the desired range by introducing carbon dioxide gas. In essence, the flow of carbon dioxide can be adjusted by using an electro or selenoid valve piloted by the controller. During the natural release of CO<sub>2</sub> gas out of the calcifying solution, the pH will increase to about 6-10, more preferably about 7.5 to 8.5 after soaking for 24 hours. The carbonated calcium phosphate layer comprising the protein will precipitate on the surface of implantable devices at a pH value of within about 6.5-7.5. The said precipitation on the surface of medical implants is related to a heterogeneous nucleation step. The carbonated calcium phosphate crystals might subsequently precipitate into the calcifying solution by a crystal growth process. In accordance with the invention, heterogeneous nucleation is favored by the energetic stabilization of nucleus on the substrate. The high density of nucleation ensures a uniform deposition of carbonated calcium phosphate crystals onto the surface of medical implants.

In a method according to the invention, it may be desired to control the pH and thereby the nucleation stage by bubbling CO<sub>2</sub> gas for various time. The bubbling time is usually comprised between a few seconds to minutes, preferably about 1 to 600 seconds. The introduction of carbon dioxide causes a decrease of pH while the pH of calcifying solution has a tendency to increase naturally without bubbling CO<sub>2</sub> gas. The increase of pH may be due to the natural exchange of CO<sub>2</sub> gas with atmosphere and the buffering

capability of the calcification solution. By adjusting the time and flow of CO<sub>2</sub> gas introduced into the calcifying solution, the pH can oscillate around a value ranging from 6 to 9, more preferably the pH of the calcifying solution can  
5 be maintained between 6.5 to 7.5. This pH oscillation is correlated to the nucleation stage of carbonated calcium phosphate crystals on the surface of medical implants. A high density of nucleation is thereby provided and carbonated calcium phosphate crystals can nucleate and grow onto the  
10 surface of medical implants. Homogeneous layers comprising ceramic material and protein can uniformly deposit on the implant substrate. The total thickness of layers will preferably be within the range 0.5-100 microns, more likely 0.5 to 50 microns. While the layers are thin, usually below 5  
15 microns, the coatings can diffract the natural light forming colored fringes ranging from blue to red colors. This diffraction of light is similar to the phenomenon that may be observed when a drop of oil is present on water. For higher thickness, the layers give a shiny gray or white coloration.

20           The thus obtained coating comprises both ceramic material and the protein. In accordance with the invention, the ceramic material is removed by dissolution in a second, solution to provide the objective proteinaceous coating. The conditions under which the dissolution is carried out are to  
25 be chosen such that the ceramic material substantially completely dissolves and the protein substantially completely remains coated on the surface of the medical implant. In other words, care should be taken that hydrolyzation of the protein is avoided as much as possible. Although it may be  
30 expected that a (partial) denaturation of the protein takes place during this step, this is considered not to harm the inductive properties of the coating, and may in fact even be beneficial.

          The dissolution may be accomplished by using an  
35 acidic solution, such as an aqueous solution of which the pH is preferably chosen in the range of from 2 to 5, more

preferably from 3 to 4. The acid used for obtaining the objective pH can in principle be any acid suitable for attaining the desired degree of acidity. Suitable acids do not adversely affect the protein. Examples are hydrogen  
5 phtalate and hydrochloric acid, which acids are preferably used in aqueous solutions of 30-70 mM.

In an other embodiment the dissolution of the ceramic material is accomplished in a solution comprising a complexing or sequestering agent to redissolve the magnesium,  
10 calcium and phosphate ions and to obtain the proteinaceous coating. The solution comprising the sequestering agent is preferably an aqueous solution of which the pH may be alkaline, neutral or acidic. The pH is preferably in the range of 2-9, more preferably in the range of 2-7 and. Most  
15 preferably the pH is in the range of 4-6, because of the better solubility of free calcium and magnesium at an acidic pH, in combination with a relatively high degree of complexation.

In principle any sequestering agent for calcium and  
20 magnesium can be used to dissolve the mineral part of the coating. A suitable sequestering agent does not adversely affect the protein. The sequestering agent may be used alone or together with one or more other sequestering agents, e.g. to optimize the complexation of the different cations in the  
25 ceramic materials.

Very good results have been achieved with ethylene diamine tetra acetate (EDTA, e.g. the sodium salt) as a sequestering agent. A highly preferred pH for this particular embodiment is in the range of 4-6.

30 It has been found that ceramic material can be dissolved from the coating by using a sequestering agent in a wide concentration range. A sequestering agent may be used in any concentration, up to its saturated concentration. Preferably the concentration is between 0.1 and 20 wt. %,   
35 more preferably between 1 and 10 %.

Dissolution of the ceramic material by using a sequestering or complexing agent can be performed such that the protein does not significantly denature, which can be advantageous for particular applications.

5           Once the ceramic material has essentially completely been removed, the medical implant with the proteinaceous coating may be taken out of the solution in which the ceramic material has been dissolved and optionally rinsed with water or demineralized water in order to remove trace amounts of  
10 acid and/or ceramic material. The medical implant thus obtained has been found to induce mineralization, calcifications and/or formation of bone tissue *in vitro* and *in vivo*. Accordingly, the implant has an improved performance as a bone substitute in comparison with the implant without  
15 the proteinaceous coating.

          It has also been found that upon exposure to a calcifying solution, recalcification of the proteinaceous layer may take place in such a way that the morphology of the layer after remineralization closely resembles that of the  
20 intermediate coating, comprising protein and calcium phosphate, before the decalcification. This recalcification process is expected to take place *in vivo*, depending on the site of implantation. Since the layer may completely resorb the minerals, the risk for creating brittle zones in the  
25 coatings that may cause fracture, is minimized.

          Furthermore, the inductive property of the coating makes the coated implant highly suitable for use as a scaffold for tissue engineering bone. In this application, cells may be seed onto the implant and cultured to form or to  
30 start forming bone tissue.

          The invention will now be elucidated by the following, non-restrictive examples.

### EXAMPLE

#### **Materials and Methods**

##### **Materials**

5 High-grade titanium alloy (Ti6Al4V) plates of 20 x 20  
x 1 mm were used. The calcifying solutions were prepared with  
reagent pure chemicals (Merck) and demineralized water. The  
protein used for this study was bovine serum albumin (BSA) in  
a powder form (fraction V, >98%, Sigma). BSA is a single  
10 polypeptide chain consisting of about 583 amino acid residues  
(M.W. 66,4303). At pH 5-7, it contains 17 intra-chain  
disulfide bridges and 1 sulfhydryl group (Sigma A-8806)

##### **Methods**

#### **Preparation of Biomimetic Calcium Phosphate Coatings**

15 Ti6Al4V plates were ultrasonically cleaned for 15 min  
in acetone, then ethanol (70 %) and finally demineralized  
water. All the samples were etched with a mixture of  
hydrofluoric acid (HF, 40 wt.%) nitric acid (HNO<sub>3</sub>, 65 wt.%)  
20 and demineralized water for 10 min under ultrasounds. After  
etching, they were thoroughly rinsed with demineralized  
water.

Two layers of calcium phosphate were subsequently  
applied on the Ti6Al4V samples by using a biomimetic method.  
25 The first layer was prepared at high nucleation conditions in  
the presence of inhibitors of crystal growth under a  
concentrated Simulated Body Fluid (SBFx5) (Table 1). A  
calcifying solution was made by dissolving NaCl, CaCl<sub>2</sub>.2H<sub>2</sub>O,  
MgCl<sub>2</sub>.6H<sub>2</sub>O, NaHCO<sub>3</sub> and Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O salts in 1000 ml of  
30 demineralized water and passing through carbon dioxide gas.  
After immersion for 24 hours at 37°C, pH of this solution has  
increased from 5-6 till 8. The plates were carefully rinsed  
with demineralized water for 10 minutes and finally dried at  
room temperature overnight. A thin, dense and amorphous  
35 calcium phosphate layer was uniformly deposited on the  
titanium alloy surface. This thin layer diffracted natural

light forming colorful fringes and was so-called Rainbow coating. The Rainbow coated Ti6Al4V plates were used as seed surface for growing a subsequent crystalline apatite layer.

Table 1:

Solution	Ionic concentration (mM)								
	Buffer	Na <sup>+</sup>	K <sup>+</sup>	Mg <sup>2+</sup>	Ca <sup>2+</sup>	Cl <sup>-</sup>	HPO <sub>4</sub> <sup>2-</sup>	SO <sub>4</sub> <sup>2-</sup>	HCO <sub>3</sub> <sup>2-</sup>
HBP	-	142.0	5.0	1.5	2.5	147.8	1.0	0.5	4.2
SBF x5	CO <sub>2</sub>	733.5	-	7.5	12.5	739.0	5.0	2.5	21.0
CPS	Tris-HCl	140.0	-	-	4.0	144.0	2.0	-	-
SPS	KH <sub>2</sub> PtA- late-HCL	137.0	-	-	-	177.0	-	-	-

5 HBP = a typical composition of Human Blood Plasma

The second layer was prepared under conditions conducive to crystal growth, namely, by immersing Ti6Al4V implants in supersaturated calcium phosphate (CPS, Table 1) solution for 48 hours at ambient temperature. These solutions also contained bovine serum albumin (Bovine serum albumin (BSA) in a powder form, fraction V, >98%, Sigma) at concentrations of 0, 0.01, 0.1 and 1 mg/ml BSA.

15 Samples were then washed in demineralized water and air-dried at ambient temperature. Scanning Electronic Microscope (SEM, Philips, Model 525, 15 kV, carbon sputtered samples) revealed the protein films to be leaf-like structures, repeating the original lamellar hydroxyapatite crystals (Figure 1a). During this coating process, a thick (30 - 50  $\mu$ m) and dense crystallized apatite film was deposited uniformly upon the surface of the substrate. The "Rainbow" layer acts as a seed structure and is resorbed during this step.

25 Treatment of these coated implants with acidic solutions (SPS, Table 1) at 37°C led to the complete dissolution of crystallized mineral components (calcium and phosphate) (13), a fine (7 - 10  $\mu$ m), soft and porous (Figure 1b) protein matrix being left behind.

30 Scanning electron microscopy revealed the remineralization process to have converted the protein layer back to the original lamellar film morphology. Some

overgrowth of rose-like crystals clusters was also seen on top of the films (Figure 1c). Implants devoid of a protein film did not become coated with a mineralized layer.

Fourier Transform InfraRed spectrometry (FT-IR, Perkin-Elmer, Spectrum 1000) using transparent KBr pellets) of the original mineralized film clearly shows the characteristic phosphate doublet at 563 and 602  $\text{cm}^{-1}$  (figure 2a). This doublet absent after demineralization (figure 2b). The phosphate doublet re-appears after remineralization (figure 2c). The BSA shows a large peak due to OH, >NH and absorbed water around 3500  $\text{cm}^{-1}$  and characteristic bands of >C=O and -COO<sup>-</sup> at 1640  $\text{cm}^{-1}$  and 1550  $\text{cm}^{-1}$ .

The protein films were insoluble in solution of both acidic and neutral pH. After release of coating, thickness of BSA film was measured by using a magnetic induction probe (Electrophysik Minitest 2100). The measuring range of this apparatus is between 0 and 100 microns. The measurements were repeated 10 times and averages were obtained on each sample. Their thickness increased as the concentration of BSA in the bathing SCP solution was raised (Table 2).

Table 2: Properties of the protein film for various protein concentrations in the mineralization solution

Concentrations of BSA in coating solution (mg/ml)	Thickness of the remineralized coating ( $\mu\text{m}$ ) (SD)	Thickness of the protein film ( $\mu\text{m}$ ) (SD)	Pore size ( $\emptyset$ ) of the protein film ( $\mu\text{m}$ ) (SD)
0.01	34.52 (5.5)	6.72 (1.67)	9.07 (1.30)
0.1	38.75 (7.32)	7.80 (1.72)	2.67 (1.08)
1.0	37.25 (7.70)	10.00 (1.06)	1.07 (0.25)

25

The pores between the crystals remain as pores in the demineralized protein film (Table 2). Crystal size - and hence pore size - decreased as the concentration of BSA in the SCP solution was elevated. The thickness of the

30



demineralized protein film increased from about 20% to 27% of the precursor mineralized film with increasing BSA concentration. No residual apatite crystals were seen in these protein films.

- 5 Energy dispersive x-ray analysis (EDS, Voyager) gave evidence of residual calcium and phosphate in the protein films being below 1.14% (Table 3).

10 Table 3: EDS comparison of atomic percentage by element in coated implants

Atomic %	C	O	Na	P	Cl	Ca	Ca/P
Apatite/BSA coating	35.28	40.75	-	10.22	0.47	13.28	1.3
"demineralized" protein matrix	65.35	24.81	3.2	1.14	2.92	0.96	0.8
"re-mineralized" protein matrix	33.32	46.83	1.68	8.55	0.78	8.38	1.0

- 15 When implants that were covered with these protein films were again immersed in SCP solutions (without BSA) under the same conditions, they became recoated with a thick (30 $\mu$ m), dense layer of apatite (deducted from results shown in Figure 3).

Claims

1. A method for providing a proteinaceous coating on a medical implant, comprising the steps of:
  - submersing the implant in a first aqueous solution comprising a protein and magnesium, calcium and phosphate
  - 5 ions through which a gaseous weak acid is passed;
  - degassing the solution;
  - allowing a coating to precipitate onto the implant;
  - submersing the coated implant in a second solution to redissolve the magnesium, calcium and phosphate ions and to
  - 10 obtain the proteinaceous coating.
2. A method according to claim 1, wherein the gaseous weak acid is carbon dioxide.
3. A method according to claim 1 or 2, wherein the implant is a metallic, organic, polymeric, or ceramic implant.
- 15 4. A method according to any of the preceding claims, wherein the calcium and phosphate ions are present in the first solution in a molar ratio of between 1 and 3, preferably between 1.5 and 2.5.
5. A method according to claim 4, wherein the first
- 20 solution comprises 0.5-50 mM, preferably 2.5-25 mM, calcium ions, and 0.5-20 mM, preferably 1-10 mM phosphate ions.
6. A method according to any of the preceding claims, wherein the first solution comprises 0.1-20, preferably 1.5-10 mM magnesium ions.
- 25 7. A method according to any of the preceding claims, wherein the first solution further comprises 0-50 mM, preferably 0-42 mM carbonate ions.
8. A method according to any of the preceding claims, wherein the ionic strength of the first solution is in the
- 30 range of 0.1-2 M, preferably of 0.15-1.5 M.
9. A method according to any of the preceding claims, wherein the pressure of the gaseous weak acid is in the range of 0.1-10 bar, preferably of 0.5-1.5 bar.

10. A method according to any of the preceding claims, wherein the temperature of the first and second solutions are independently chosen in the range of 5 to 80°C, preferably of 5 to 50°C.
- 5 11. A method according to any of the preceding claims, wherein the protein is chosen from the group of albumin, casein, gelatin, lysosime, fibronectin, fibrin, chitosan, polylysine, polyalanine, polycysteine, growth factors, and combinations thereof.
- 10 12. A method according to any of the preceding claims, wherein the protein is present in the first solution in a concentration of between 0.001 and 10 g/l.
13. A method according to any of the preceding claims, wherein the second solution is an aqueous acidic solution.
- 15 14. A method according to claim 13, wherein the second solution has a pH of between 2 and 5.
15. A method according to any of the preceding claims, wherein the second solution comprises a sequestering or complexing agent.
- 20 16. A method according to claim 15, wherein the sequestering or complexing agent is ethylene diamine tetra acetate.
17. A method according to claim 15 or 16, wherein the sequestering agent is present in a concentration between 0.1 and 20 wt.%.
- 25 18. A medical implant comprising a proteinaceous coating obtainable by a method according to any of the preceding claims.
19. A medical implant according to claim 18, wherein the proteinaceous coating has a thickness in the range of from
- 30 0.5 to 100 microns.
20. Use of a medical implant according to any of the claims 18-19 as a scaffold for tissue engineering bone.
21. Use of a proteinaceous coating obtainable by a method according to any of the claims 1-17 for inducing
- 35 mineralization and/or formation of bone tissue.

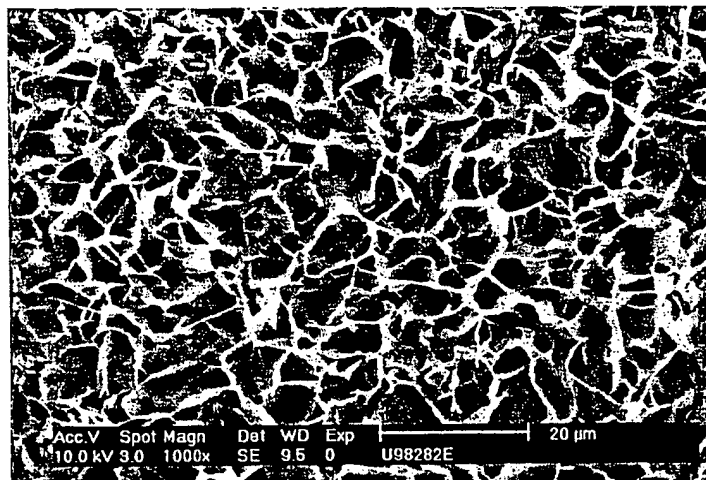


Fig 1a: Ca-P/BSA coating

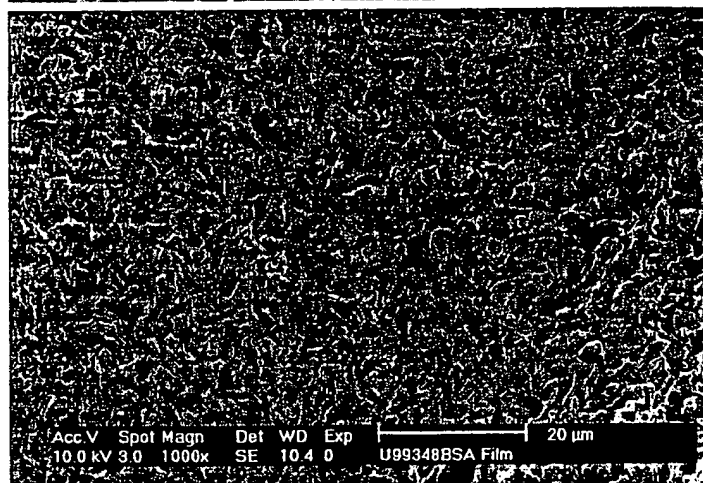


Fig 1b: "demineralized"



Fig. 1c: "remineralized"

Figure 2a

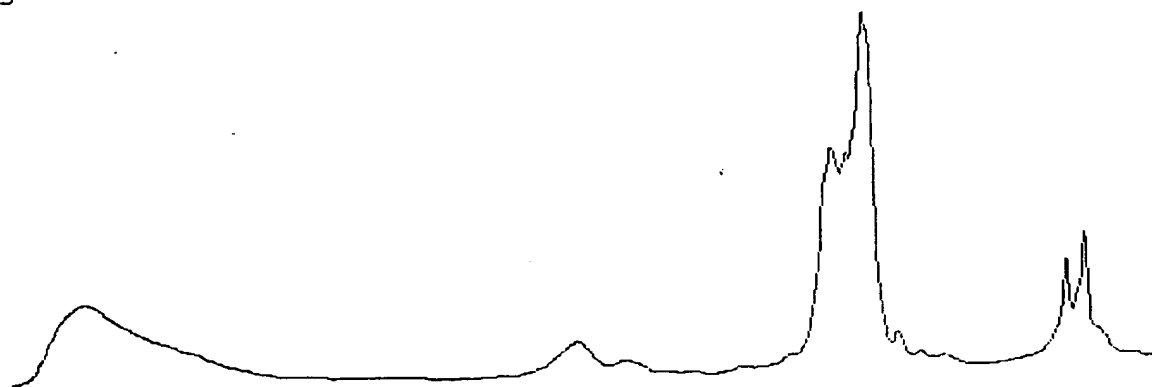


Figure 2b

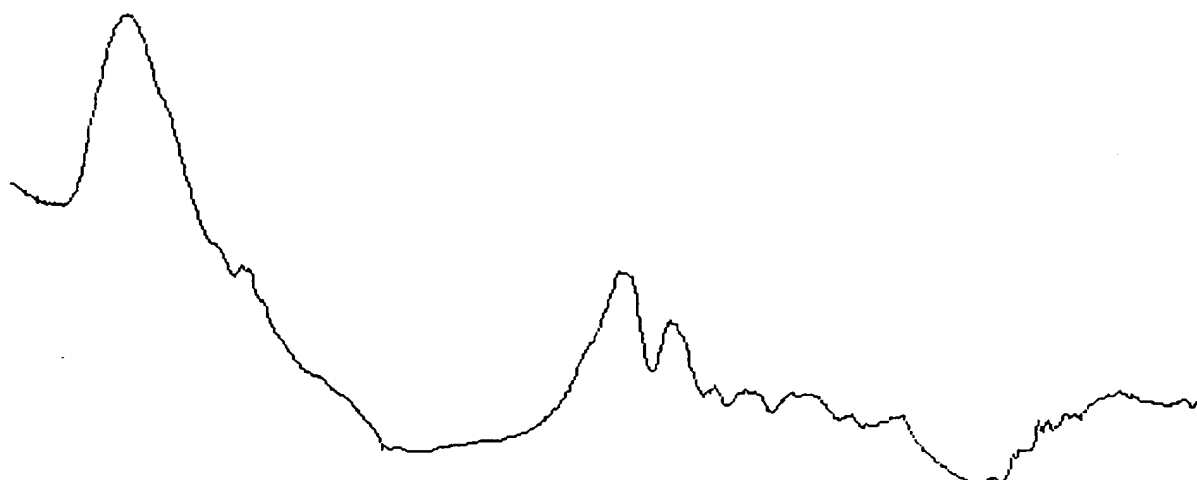


Figure 2c

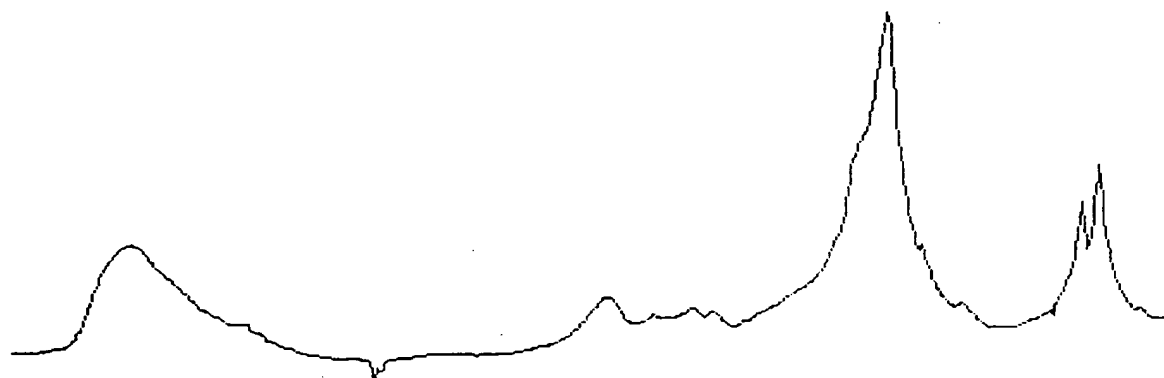
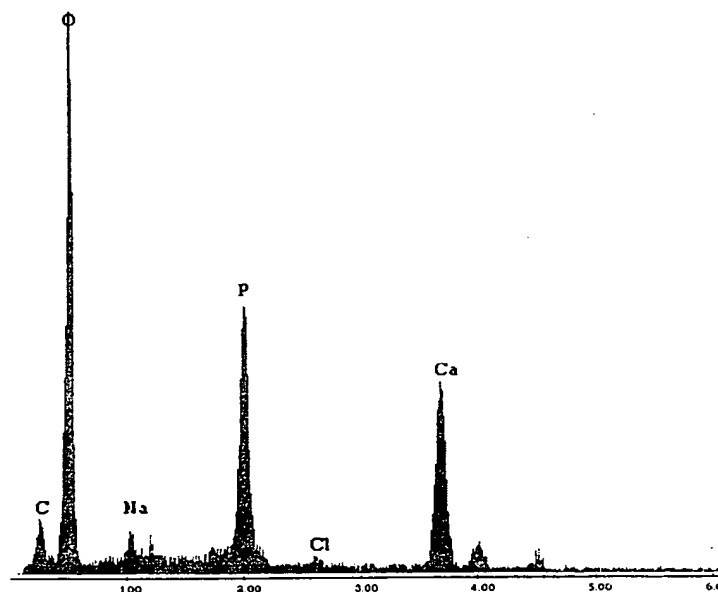


Figure 3: EDS of remineralized protein matrix



# INTERNATIONAL SEARCH REPORT

In tional Application No  
PCT/NL 01/00071

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61L27/34 A61L31/10 A61L27/32 A61L31/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 806 212 A (MATRIX MEDICAL B V) 12 November 1997 (1997-11-12) cited in the application page 3, line 3 - line 31; claims ---	1-14, 18-21
Y	WO 97 41273 A (BIOTECNIC S A ;RANZ XAVIER (FR); REY CHRISTIAN (FR); 3C RESEARCH S) 6 November 1997 (1997-11-06) cited in the application page 5, line 18 - line 20; claims page 10, line 15 - line 18; claims ---	1-14, 18-21
A	US 4 713 076 A (DRAENERT KLAUS) 15 December 1987 (1987-12-15) claims; example --- -/--	1-14, 18-21



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&\* document member of the same patent family

Date of the actual completion of the international search

28 May 2001

Date of mailing of the international search report

12/06/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5618 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

ESPINOSA, M

# INTERNATIONAL SEARCH REPORT

In tional Application No

PCT/NL 01/00071

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 11202 A (ICET INC) 11 March 1999 (1999-03-11) claims; examples ---	1-14, 18-21
A	WO 95 13101 A (GROOT KLAAS DE) 18 May 1995 (1995-05-18) claims; examples 1,2 ---	1-14, 18-21
A	WO 99 45979 A (JOHNSON & JOHNSON PROFESSIONAL) 16 September 1999 (1999-09-16) page 10, line 30 - line 34; claims -----	1-14, 18-21



# INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No

PCT/NL 01/00071

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0806212 A	12-11-1997	CA 2205104 A	10-11-1997
		CA 2205107 A	10-11-1997
		EP 0806211 A	12-11-1997
		US 6136369 A	24-10-2000
		US 6146686 A	14-11-2000
		US 6069295 A	30-05-2000
		US 6143948 A	07-11-2000
WO 9741273 A	06-11-1997	IT PR960021 A	30-10-1997
		DE 69702610 D	24-08-2000
		DE 69702610 T	08-03-2001
		EP 0904421 A	31-03-1999
US 4713076 A	15-12-1987	DE 3414924 A	31-10-1985
		AT 79009 T	15-08-1992
		DE 3586438 A	10-09-1992
		EP 0159036 A	23-10-1985
		JP 1840105 C	25-04-1994
		JP 5052225 B	04-08-1993
		JP 61033660 A	17-02-1986
WO 9911202 A	11-03-1999	US 6129928 A	10-10-2000
WO 9513101 A	18-05-1995	NL 9301941 A	01-06-1995
WO 9945979 A	16-09-1999	US 6139585 A	31-10-2000
		AU 2991999 A	27-09-1999
		EP 0984799 A	15-03-2000